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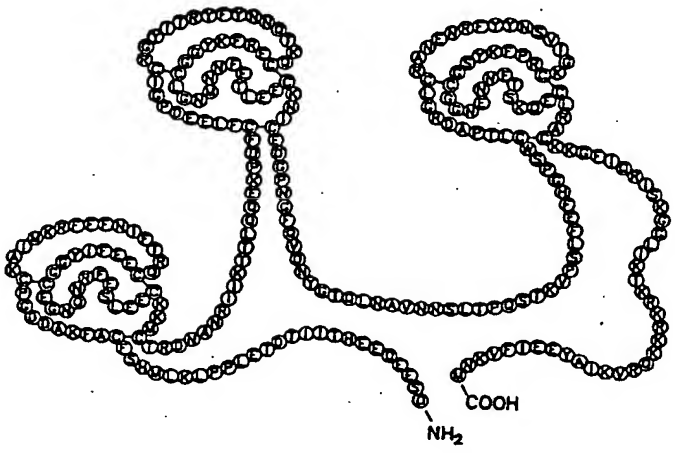

(57) Abstract

Novel EPI analogues are provided wherein one or more of the amino acid residues of native EPI have been deleted. A preferred group of the novel EPI analogues have no or a low heparin binding capacity. The novel EPI analogues can be used for the treatment of patients having coagulation disorders or cancer.

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ANTICOAGULANT PROTEIN

Field of the invention

The present invention relates to a new protein with EPI activity, a method of producing such protein and a therapeutic preparation containing such protein.

Background of the invention

Blood coagulation is a complex process involving many activating and inactivating coagulation factors. Anticoagulant proteins are known to be important for regulation of the coagulation process (see B. Lämmle and J. Griffin (Clinics in Haematology 14 (1985), 281-342) and anticoagulants are thus important in the treatment of a variety of diseases, eg thrombosis, myocardial infarction, disseminated intravascular coagulation etc.

Thus heparin is used clinically to increase the activity of antithrombin III and heparin cofactor II. Antithrombin III is used for the inhibition of factor Xa and thrombin. Hirudin is used for the inhibition of thrombin and protein C may be used for the inhibition of factor V and factor VIII.

Anticoagulant proteins may also be used in the treatment of cancer. Thus, antistatin has been shown to have anti-metastatic properties (J.H. Han et al., Gene 75 (1989), 47-57). Also heparin and warfarin have been shown to possess antimetastatic properties (G.J. Gasic et al., Int. Rev. Exp. Pathol. 29 (1985), 173-209).

Coagulation can be initiated through the extrinsic pathway by the exposure of tissue factor (TF) to the circulating blood (Y. Nemerson, Blood 71 (1988), 1-8). Tissue factor is a protein cofactor for factor VII/VIIa and binding of tissue factor enhances the enzymatic activity of factor VIIa (FVIIa) towards

its substrates factor IX and factor X. Placenta anticoagulant pr tein is able to inhibit tissue factor activity, probably by interfering with TF/FVIIa-phospholipid interaction (S. Kondo et al., Thromb. Res. 48 (1987), 449-459).

Recently a new anticoagulant protein, the extrinsic pathway inhibitor (EPI) has been isolated (Broze et al., Proc. Natl. Acad. Sci. 84 (1987), 1886-1890).

On a molar basis EPI has been shown to be a potent inhibitor of TF/FVIIa induced coagulation (R.A. Gramzinski et al., Blood 73 (1989), 983-989). EPI binds and inhibits factor Xa (FXa) and the complex between EPI and FXa inhibits TF/FVIIa (Rapaport, Blood 73 (1989), 359-365). EPI is especially interesting as an anticoagulant/antimetastatic agent as many tumor cells express TF activity (T. Sakai et al., J. Biol. Chem. 264 (1989), 9980-9988) and because EPI shows anti-Xa activity like antistatin.

EPI has been recovered by Broze et al. (supra) from HepG2 hepatoma cells (Broze EP A 300988) and the gene for the protein has been cloned (Broze EP A 318451). A schematic diagram over the secondary structure of EPI is shown in Fig. 1 and the amino acid sequence of EPI is shown in Fig. 2 where the N-terminal amino acid Asp is given the number 1. The protein consists of 276 amino acid residues and has in addition to three inhibitor domains of the Kunitz type three potential glycosylation sites at position Asn117, Asn167 and Asn229. The molecular weight shows that some of these sites are glycosylated. Furthermore, it has been shown that the second Kunitz domain binds FXa while the first Kunitz domain binds FVIIa/TF (Girard et al., Nature 338 (1989), 518-520). EPI has also been isolated from HeLa cells (PCT/DK90/00016) and it was shown that HeLa EPI binds heparin.

Heparin binding is an important factor for the pharmacokinetics of substances for injecti n. It has b en shown that platelet factor 4 (M. Prosdomi et al., Thromb. Res. 39 (1985), 541-

547) and aprotinin with one Kunitz domain (H. Fritz et al., Drug Res. 33, 479-94) has a sh rt half life probably du to the heparin binding properties. Lowering of the heparin binding capacity of an anticoagulant would therefore seem to be advantagous. Furthermore, it might be advantagous to use a smaller molecule than EPI for the medical treatment.

It has according to the present invention surprisingly been found that certain EPI analogues retain the EPI activity as well as anti Xa activity although parts of the molecule has been deleted. Furthermore, these analogues show a much lower affinity for heparin than full length EPI, making them more useful as therapeutic agents than the native molecule. The EPI analogues will furthermore have a longer half life as compared with native EPI which will further reduce the amount of active ingredients for the medical treatment.

Summary of the invention

In its first aspect the present invention is related to novel EPI analogues wherein one or more of the amino acid residues of native EPI have been deleted.

In its second aspect the present invention is related to a new group of EPI analogues having EPI activity but with no or low heparin binding capacity under physiological conditions (pH, ionic strength).

In the present context the term "low heparin binding capacity" is intended to mean a binding capacity of about 50%, more preferably of about 25% and most preferably less than about 10% of that of native EPI at physiological pH and ionic strength.

The preferred group of the novel EPI analogues can be characterized as being devoid of the heparin binding domain of native EPI or having a non-functional heparin binding domain by having deleted one or more of the amino acid residues in said

domain resulting in loss or a substantial lowering of the heparin binding capacity. The same effect may also be obtained by substituting one or more of the amino acid residues in the heparin binding domain with another amino acid residue.

Detailed description of the invention

To retain the EPI acitivity the analogues according to the present invention should at least contain the N-terminal sequence including the first and second Kunitz domain. Thus, the EPI analogues according to the present invention should at least contain the amino acid sequence from amino acid number 25 to amino acid number 148 of native EPI (see fig. 1 and 2).

It has been shown by the inventors hereof that the heparin binding capacity is lost when the sequence from amino acid residue number 162 to amino acid residue number 275 is deleted from the EPI molecule. It is therefore concluded that the heparin binding domain is situated in this part of the EPI molecule. It is assumed that the heparin binding domain comprises at least a region from Arg246 to Lys265 near the C-terminal end of the EPI molecule being rich in positively charged amino acid residues.

Preferred EPI analogues according to the present invention are such in which one or more amino acid residues have been deleted in the native EPI molecule from amino acid residue Glu148 to the C-terminal Met276.

More specifically, one or more amino acid residues in the sequence from Arg246 to Lys275 have been deleted.

Examples of EPI analogues according to the present invention are:

(Asp1 - Thr255)-EPI
(Asp1 - Ile253)-EPI
(Asp1 - Lys249)-EPI
(Asp1 - Ser248)-EPI
(Asp1 - Lys240)-EPI
(Asp1 - Glu234)-EPI
(Asp1 - Trp188)-EPI
(Asp1 - Asn164)-EPI
(Asp1 - Thr161)-EPI
(Asp1 - Asp149)-EPI
(Asp1 - Glu148)-EPI

Ser-(Asp1 - Thr255)-EPI
Ser-(Asp1 - Ile253)-EPI
Ser-(Asp1 - Lys249)-EPI
Ser-(Asp1 - Ser248)-EPI
Ser-(Asp1 - Lys240)-EPI
Ser-(Asp1 - Glu234)-EPI
Ser-(Asp1 - Trp188)-EPI
Ser-(Asp1 - Asn164)-EPI
Ser-(Asp1 - Thr161)-EPI
Ser-(Asp1 - Asp149)-EPI
Ser-(Asp1 - Glu148)-EPI

(Glu15 - Thr255)-EPI
(Glu15 - Ile253)-EPI
(Glu15 - Lys249)-EPI
(Glu15 - Ser248)-EPI
(Glu15 - Lys240)-EPI
(Glu15 - Glu234)-EPI
(Glu15 - Trp188)-EPI
(Glu15 - Asn164)-EPI
(Glu15 - Thr161)-EPI
(Glu15 - Asp149)-EPI
(Glu15 - Glu148)-EPI

Ser-(Glu15 - Thr255)-EPI
Ser-(Glu15 - Ile253)-EPI
Ser-(Glu15 - Lys249)-EPI
Ser-(Glu15 - Ser248)-EPI
Ser-(Glu15 - Lys240)-EPI
Ser-(Glu15 - Glu234)-EPI
Ser-(Glu15 - Trp188)-EPI
Ser-(Glu15 - Asn164)-EPI
Ser-(Glu15 - Thr161)-EPI
Ser-(Glu15 - Asp149)-EPI
Ser-(Glu15 - Glu148)-EPI

(Ser24 - Thr255)-EPI
(Ser24 - Ile253)-EPI
(Ser24 - Lys249)-EPI
(Ser24 - Ser248)-EPI
(Ser24 - Lys240)-EPI
(Ser24 - Glu234)-EPI
(Ser24 - Trp188)-EPI
(Ser24 - Asn164)-EPI
(Ser24 - Thr161)-EPI
(Ser24 - Asp149)-EPI
(Ser24 - Glu148)-EPI

(Asp1 - Thr255)-(Ile266 - Met276)-EPI
(Asp1 - Ile253)-(Ile266 - Met276)-EPI
(Asp1 - Ser248)-(Ile266 - Met276)-EPI
(Asp1 - Gln245)-(Ile266 - Met276)-EPI
(Asp1 - Thr255)-(Val264 - Met276)-EPI
(Asp1 - Ile253)-(Val264 - Met276)-EPI
(Asp1 - Ser248)-(Val264 - Met276)-EPI
(Asp1 - Glu245)-(Val264 - Met276)-EPI
(Asp1 - Thr255)-(Glu262 - Met276)-EPI
(Asp1 - Ile253)-(Glu262 - Met276)-EPI
(Asp1 - Ser248)-(Glu262 - Met276)-EPI
(Asp1 - Glu245)-(Glu262 - Met276)-EPI

Ser-(Asp1 - Thr255)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Ile253)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Ser248)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Gln245)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Thr255)-(Val264 - Met276)-EPI
Ser-(Asp1 - Ile253)-(Val264 - Met276)-EPI
Ser-(Asp1 - Ser248)-(Val264 - Met276)-EPI
Ser-(Asp1 - Glu245)-(Val264 - Met276)-EPI
Ser-(Asp1 - Thr255)-(Glu262 - Met276)-EPI
Ser-(Asp1 - Ile253)-(Glu262 - Met276)-EPI
Ser-(Asp1 - Ser248)-(Glu262 - Met276)-EPI
Ser-(Asp1 - Glu245)-(Glu262 - Met276)-EPI

In addition to the described deletions in the EPI molecule, certain amino acid residues of native EPI may also be replaced by another naturally occurring amino acid residue. The EPI analogues may also advantageously contain a Ser residue as the N-terminal residue. This is necessary if a signal sequence is used requiring an N-terminal Ser in the mature protein as a recognition site for cleavage. Thus, the N-terminal in the EPI molecule may be replaced by a Ser or an additional Ser may be inserted adjacent to the original N-terminal residue. Also the potential glycosylation sites at Asn167 and Asn238 may be substituted by another amino acid residue to avoid glycosylation.

It has not previously been shown that glycosylation sites are dispensable for EPI activity. Neither has it been shown that large fragments of the EPI protein are dispensable for EPI activity, i.e. for FXa dependent inhibition of TF/FVIIa. It has previously been shown that a single amino acid in EPI can be replaced by another amino acid residue (Arg199 -> Leu199), (Girard et al., Nature 338 (1989), 518-520) without affecting the activity. However, such a change does not significantly change the structure of the protein. In contrast hereto the deletions according to the present invention give rise to new

molecules with other properties than the native molecule, as illustrated by the changed affinity for heparin.

The present EPI-analogues may be produced by well-known recombinant DNA technology.

The gene for the native EPI has been cloned and sequenced (Wun et al., J. Biol. Chem. 263 (1988), 6001-6004). DNA-sequences encoding the desired EPI-analogue may be constructed by altering EPI cDNA by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures.

The DNA sequence encoding the EPI analogue of the invention may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

In a further aspect, the present invention relates to a recombinant expression vector which comprises a DNA sequence encoding the EPI analogue of the invention. The expression vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genom and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the EPI analogue of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the EPI analogue of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol.Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814), the adenovirus 2 major late promoter or the CMV (cytomegalovirus IE1) promoter (Henninghausen et al., EMBO J. 5 (1986), 1367-71). Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J.Biol.Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J.Mol.Appl.Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al., eds.), Plenum Press, New York, 1982), or the PTI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tipA promoter.

To ensure secretion a suitable signal sequence is inserted at the 5' of the DNA sequence encoding the EPI analogue. A suitable signal sequence is the t-PA signal sequence (Friezner et al., J.Biol.Chem. 261 (1986), 6972-85).

The DNA sequence encoding the EPI analogue of the invention should also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation

signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g neomycin, hygromycin or methotrexate.

The procedures used to ligate the DNA sequences coding for the EPI analogue of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op. cit.).

In a further aspect, the present invention relates to a cell which contains the recombinant expression vector described above. The host cell may be any cell which is capable of producing the EPI analogue and is preferably a eukaryotic cell, in particular a mammalian cell. Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J.Mol.Biol. 159, 1982, pp. 601-621; Southern and Berg, J.Mol.Appl.Genet. 1, 1982, pp. 327-341; Loyter et al., Proc.Natl.Acad.Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neuman et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells (including yeast cells) may be used as host cells of the invention. Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of Saccharomyces cerevisiae. Examples of other fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 272,277.

In a still further aspect the present invention relates to a process for producing an EPI analogue according to the invention, which comprises culturing a cell as described above in a suitable nutrient medium under conditions which are conductive to the expression of the EPI analogue, and recovering the polypeptide from the culture. The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The EPI analogue will preferably be secreted to the growth medium and may be recovered from the medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

The novel EPI analogues may be used for the treatment of patients having coagulation disorders or cancer.

Accordingly the invention is also related to a pharmaceutical preparation for the treatment of patients having coagulation disorders or cancer containing an EPI analogue in a suitable amount together with suitable adjuvants and additions.

The pharmaceutical preparations may be in a buffered aqueous solution with appropriate stabilizers and preservatives. The solution may be heat treated and may be contained in ampoules or in carpoules for injection pens. Alternatively the stabilized solution may be freeze dried and contained in ampoules or in two chamber injection systems with freeze dried substanse in one chamber and solvent in the other chamber.

Brief description of the Drawings

The present invention is further described with reference to the drawings in which

Fig. 1 shows the amino acid sequence and the two dimensional structure of EPI,

Fig. 2 shows the amino acid sequence of native EPI,

Fig. 3 shows plasmid pJR77 containing the t-PA signal, the human cytomegalovirus IE1 gene promoter and the human growth hormone gene polyadenylation signal,

Fig. 4 shows pCMVEPIhGH containing the EPI gene,

Fig. 5 shows plasmid pEP1b1 containing a DNA sequence encoding Ser-(Thr88 - Thr161)-EPI,

Fig. 6 shows plasmid PEP1ab containing a DNA sequence encoding Ser-(Glu15 - Thr161-EPI,

Fig. 7 shows the DNA sequence for Ser-(Thr88 - Thr161)-EPI preceded by the t-PA signal sequence,

Fig. 8 shows the DNA-sequenc for the Ser-(Glu15 - Thr161)-EPI preceded by the t-PA signal sequence,

Fig. 9 shows plasmid PEPIab2 containing a DNA sequence encoding (Asp1 - Thr161)-EPI, and

Fig. 10 shows the DNA sequence for (Asp1 - Thr161)-EPI preceded by the EPI signal.

The invention is further described in the following examples which are not in any ways intended to limit the scope or spirit of the invention as claimed.

Experimental Part

Assay for EPI activity: EPI was measured in a chromogenic microplate assay, modified after the method of Sandset et al., (Thromb. Res. 47 (1989), 389-400). Heat treated plasma pool was used as a standard. This standard is set to contain 1 U/ml of EPI activity. Standards and samples were diluted in buffer A (0.05 M tris / 0.1 M NaCl / 0.1 M Na-citrate / 0.02% NaN_3 / pH 8.0) containing 2 µg/ml polybrene and 0.2% bovine serum albumin. FVIIa/TF/FX/$CaCl_2$ combination reagent was prepared in buffer A and contained 1.6 ng/ml FVIIa (Novo-Nordisk a/s), human tissue factor diluted 60 fold (Hjort, Scand. J. Clin. Lab. Invest. 9 (1957), 50 ng/ml FX (Sigma) and 18 mM $CaCl_2$. The assay was performed in microplate strips at 37°C. 50 µl of samples and standards were pipetted into the strips and 100 µl combination reagent was added to each well. After 10 minutes incubation, 25 µl of FX (3.2 µg/ml) was added to each well and after another 10 minutes 25 µl of chromogenic substrate for FXa (S2222) was added 10 minutes after the addition of substrate. The reaction was stopped by addition of 50 µl 1.0 M citric acid pH 3.0. The microplate was read at 405 nm.

Assay for anti Xa activity: HeLa EPI purified on heparin sepharose (PCT/DK90/00016) was used as a standard. This standard was assigned an amount of Xa inhibition units corresponding to the amount of EPI units measured in the EPI assay. Samples and standards were diluted in 50 mM tris/0.2% bovine serum albumin pH 7.3. 100 µl of diluted samples and standards were incubated 30 minutes at 37°C with 100 µl FXa (Stago, 14 ng/ml). 25 µl of S2222 (2 mg/ml) was added after another 2 hours at 37°C. The assay was stopped and read like the EPI assay.

Synthetic oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystem DNA synthesizer.

M13 sequencing primers and gamma-[^{32P}]-ATP (5000 Ci/mmol, 10 mCi/ml) for labelling of primers were obtained from Amersham.

Restriction endonucleases and T4 DNA-ligase were obtained from New England Biolabs. Modified T7 DNA-polymerase (Sequenase) was obtained from United States Biochemicals. pBS+ (Stratagene) was used as cloning vector for synthetic DNA fragments.

A mammalian expression vector denoted pJR77 (fig. 3) containing the human cytomegalovirus IE1 gene promoter and the human growth hormone gene polyadenylation signal was used for expression of various EPI related proteins in COS-7 cells.

XL-1 Blue (Stratagene) a derivative of E. coli K12 was used as bacterial recipient for plasmid transformations and as host for propagation and preparation of plasmid DNA.

Green monkey kidney cell-line COS-7 (ATCC # CRL 1651) was grown in Dulbecco's modified eagle medium (DMEM) (Gibco 041-1965) + 50 µg/ml gentamycin + 110 µg/ml pyruvate + 10% fetal calf serum (FCS) or DMEM + 50 µg/ml gentamycin + 110 µg/ml pyruvate + 1% ITS^{+} (insulin, transferrin, serum albumin).

Restriction endonucleases and other enzymes were used in accordance with the manufacturers recommendations. Standard recombinant DNA-techniques were carried out as described (Maniatis et al., Molecular cloning. Cold Spring Harbor Laboratory, 1982).

DNA sequences were determined by the dideoxy chain termination method (Sanger et al., Proc. Natl. Acad. Sci. 74 (1977), 5463-67) using double stranded plasmid DNA as template and ^{32}P-labelled primers and Sequenase.

Plasmid DNA was introduced into COS-7 cells by calcium phosphate coprecipitation (Graham & van der Eb, Virology 52 (1973), 456-457).

$7x10^5$ cells were seeded in 20 cm^2 dishes in 5 ml DMEM + FCS. The following day each dish was added 20 µg plasmid DNA in 0.5 precipitate and 50 µl 10 mM chloroquine diphosphate. Cells were incubated overnight with precipitate. The following day fresh medium DMEM + ITS$^+$ was added. After two days of incubation media were harvested and assayed for EPI-activity and anti Xa-activity.

Example 1

Preparation of DNA-fragments encoding EPI and EPI analogues and transient expression in COS-7 cells.

The EPI cDNA sequence is described by Wun et al. (J. Biol. Chem. 263 (1988), 6001-6004). Based on the known sequence synthetic DNA-sequences encoding EPI and EPI analogues were constructed by silent mutations introducing restriction endonuclease recognition sites at suitable locations.

Expression cassettes encoding EPI fragments using the tPA signal sequence (fig. 7 and 8) and an expression cassette encoding the entire EPI protein were assembled by sequentially insertion into the cloning vector pBS+ of annealed complemen-

tary synthetic oligonucleotid s. Following assembly of DNA fragment sequences were verified by DNA sequencing as described.

Finally expression casettes were transferred to expression vector pJR77 giving rise to the following expression plasmids:

pEPIb1 encoding the tPA signal followed by EPI analogue Ser-(Thr88 - Thr161)-EPI.
pEPIab encoding the tPA signal followed by EPI analogue Ser-(Glu15 - Thr161)-EPI.
pCMVEPIhGH encoding the entire EPI protein.

COS-7 cells were transfected as indicated in Table 1. After 2 days of incubation with DMEM + ITS* media were harvested. Table 1 shows assay results for EPI-activity and anti-Xa activity.

Table 1.

Plasmid DNA	EPI [U/ml]	anti-Xa [U/ml]
pBS+	0.19	0.19
pCMVEPIhGH	8.3	7.6
pEPIb1	0.28	0.15
pEPIab	4.5	5.6

The results in table 1 show that the EPI-fragment Ser-(Thr88 - Thr161)-EPI containing only the second Kunitz domain has no activity whereas the EPI-fragment Ser-(Glu15 - Thr161)-EPI containing the first and second Kunitz domain has retained the EPI activity.

Example 2

COS transfections with EPI plasmids were performed as described in example 1 and media were harvested after 48 hours. 5 columns were packed, each with 300 ul of heparin sepharose. The columns were equilibrated with 20 mM tris/10% glycerol, pH 7.5. (buffer B). 1.3 ml culture medium was applied to each column. Then the columns were washed with 1.5 ml buffer B and each column was eluted with steps of 1.5 ml buffer B with increasing amounts of NaCl. For comparison is given data from another experiment where EPI from HeLa and HepG2 cells were fractionated on heparin-sepharose. In this experiment other NaCl concentrations were used for elution. Table 2 shows that Ser-(Glu15 - Thr161)-EPI does not bind to heparin at physiological pH and ionic strength.

Table 2. Heparin binding of EPI and EPI-like proteins

Culture medium	U EPI in medium	% of EPI activity flow through	B wash	M NaCl 0.25	0.5	0.75	2.0
untransfected COS	0.25	<4	<4	<4	17	54	33
EPI-transfected COS	7.10	<4	<4	<4	6	48	<4
Ser-(Glu15-Thr161)-EPI transfected COS	8.34	76	24	<3	<3	<3	<3
untransfected HeLa	1.17	NM	NM	ND	64	ND	15
untransfected HepG2	1.95	NM	NM	ND	12	ND	74

NM: not measured, ND: not done

Example 3

Preparation of (Asp1 - Thr161)-EPI

Construction of expression plasmids, transformation and expression in COS-7 cell was performed using materials and methods as described in Example 1.

Sequences between SalI and BamHI of expression plasmid pEPIab (Fig. 6) encoding the t-PA signal and Ser-(Glu15 - Thr161)-EPI was replaced by a synthetic DNA-sequence encoding the authentic EPI signal and (Asp1 - Thr161)-EPI.

The resulting plasmid pEPIab2 is shown in Fig. 9 and the expression cassette of pEPIab2 is sh wn in Fig 10.

Culture medium from pEPIab2 transfected COS cells were applied to Heparin-Sepharose as described in Example 2. 3.8 ml culture medium containing 31.2 U/ml of EPI was applied to a 0.5 ml heparin column. Flow through contained 77% and B wash contained 16% of the applied EPI activity. No EPI was detected in eulates with 0.25, 0.75 and 1.5 M NaCl respectively.

(Asp1 - Thr161)-EPI has one potential N-linked glycosylation site (Asn117) and the importance of this glycosylation for activity was investigated.

(Asp1 - Thr161)-EPI was purified from COS culture medium by affinity chromatography of FXa-Sepharose. In SDS-PAGE the purified protein appeared as a glycosylated band near 27 kDa and an unglycosylated band near 22 kDa (shown by treatment with endogylcosidase F). The glycosylated and unglycosylated forms were separated in unreduced SDS-PAGE and were extracted from the gel. Both forms were active in the EPI assay and showed the same specific activity as judged from the staining intensities in the SDS-gel. Glycosylation at Asn117 is therefore apparently not essential for EPI activity and active (Asp1 - Thr161)-EPI can thus be obtained in efficient expression systems where mammalian N-linked glycosylation is not obtained, e.g. in procaryots, or as unsecreted protein in yeast.

CLAIMS

1. EPI analogue, characterized in that it has a low heparin binding capacity or does not bind to heparin under physiological conditions.

2. EPI analogue according to claim 1, wherein the heparin binding domain has been deleted or made non-functional by deleting one or more of the amino acid residues in said domain or substituting one or more of the amino acid residues in said domain with other naturally occuring amino acid residues.

3. EPI analogue according to claim 1 or 2, characterized in that it at least contains the first and second Kunitz domain of native EPI.

4. EPI analogue according to claim 3, characterized in that it at least contains the amino acid sequence from Phe25 to Glu148 of native EPI.

5. EPI analogue according to one or more of the previous claims, wherein one or more of the amino acid residues in the sequence from Asp149 to the C-terminal Met276 of native EPI have been deleted.

6. EPI analogue according to claim 5 wherein one or more of the amino acid residues in the sequence from Arg246 to Lys265 of native EPI have been deleted.

7. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Arg249 of native EPI.

8. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Glu235 of native EPI.

9. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Thr161 of native EPI.

10. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Arg249 of native EPI.

11. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Gln235 of native EPI.

12. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Thr161 of native EPI.

13. EPI analogue according to any of the previous claims and further having a Ser as the N-terminal amino acid residue.

14. DNA-sequence encoding an EPI analogue according to any of the previous claims.

15. Expression vector containing a DNA-sequence according to claim 14.

16. Transformed or transfected microorganism or cell line comprising a vector according to claim 15.

17. Method for preparation of EPI analogues according to any of the previous claims wherein a microorganism or cell line according to claim 16 is cultured in a suitable culture medium whereupon the EPI analogue is isolated.

18. A therapeutic preparation for the treatment of patients having coagulation disorders or cancer, characterized in that it contains an EPI-analogue according to any of the previous claims 1-13 and suitable adjuvants and additions.

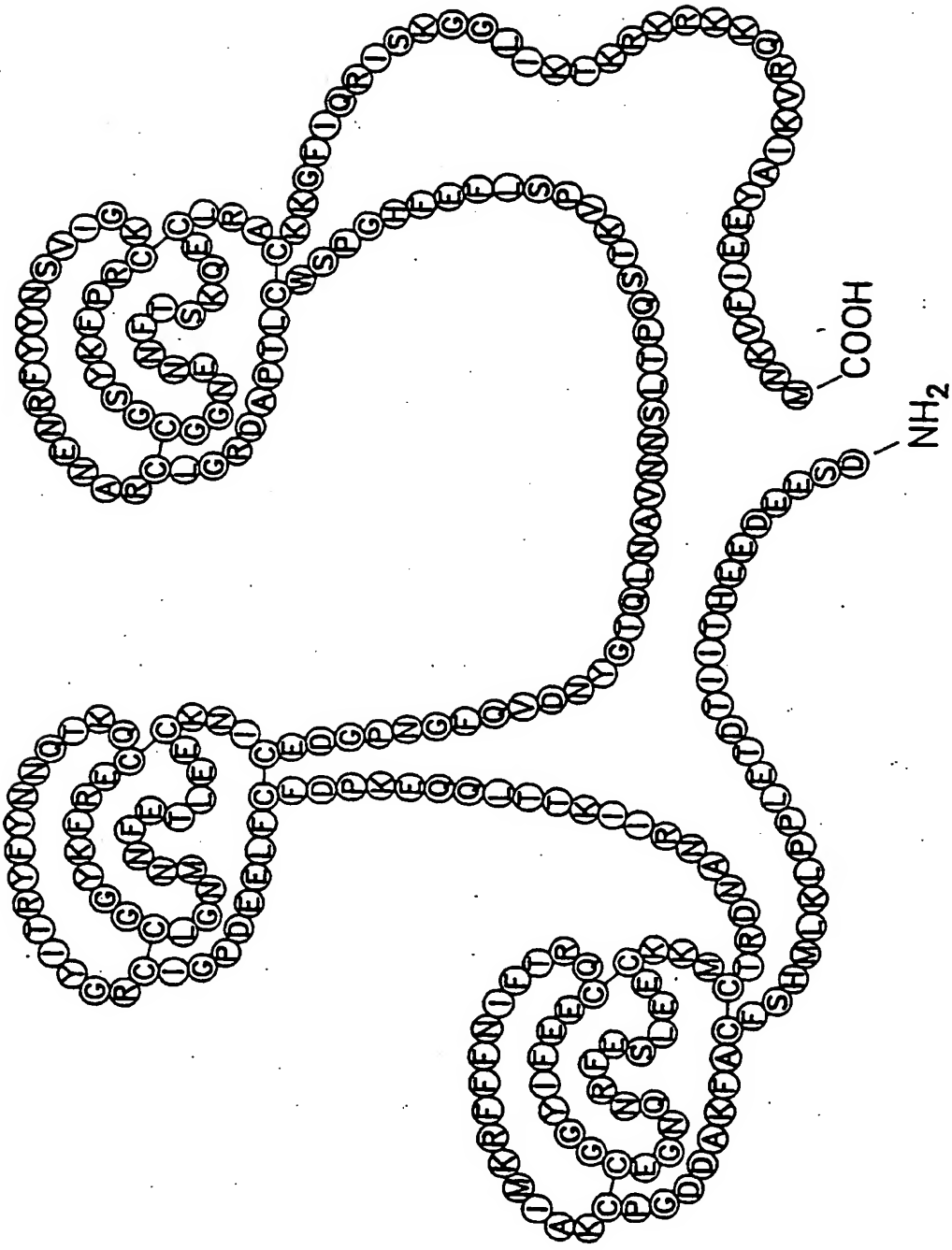

Fig. 1

```
Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu
 1               5                  10                  15

Pro Pro Leu Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp
            20                  25                  30

Gly Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Tyr
        35                  40                  45

Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn
    50                  55                  60

Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn
65                  70                  75                  80

Ala Asn Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe
                85                  90                  95

Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg
            100                 105                 110

Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly
        115                 120                 125

Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys
    130                 135                 140

Asn Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly
145                 150                 155                 160

Thr Gln Leu Asn Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys
                165                 170                 175

Val Pro Ser Leu Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro
            180                 185                 190

Ala Asp Arg Gly Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn
        195                 200                 205

Ser Val Ile Gly Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly
    210                 215                 220

Asn Glu Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys
225                 230                 235                 240

Lys Gly Phe Ile Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys
                245                 250                 255

Arg Lys Arg Lys Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe
            260                 265                 270

Val Lys Asn Met
        275
```

Fig. 2

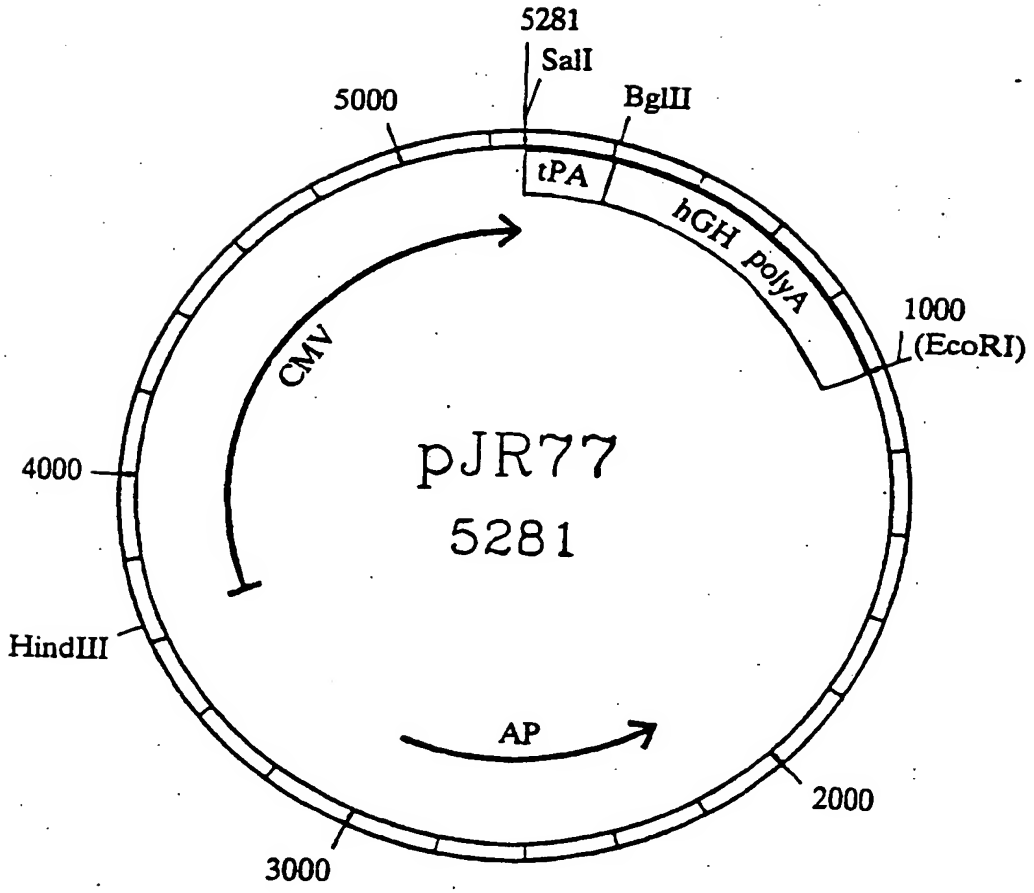
5281
SalI
BglII
5000
tPA
hGH polyA
1000
(EcoRI)
CMV
pJR77
5281
4000
HindIII
AP
2000
3000

Fig. 3

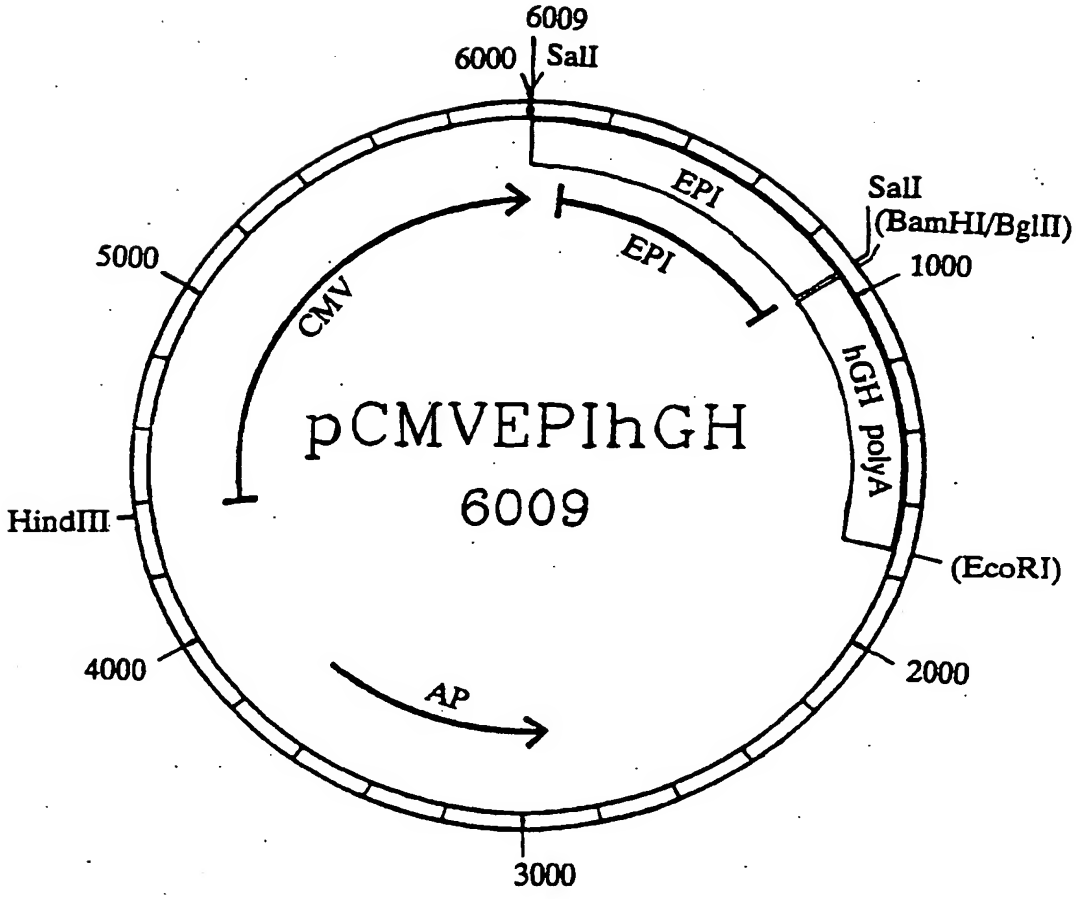
6009
6000
SalI
EPI
SalI
(BamHI/BglII)
1000
5000
EPI
CMV
hGH polyA
pCMVEPIhGH
6009
HindIII
(EcoRI)
4000
2000
AP
3000

Fig. 4

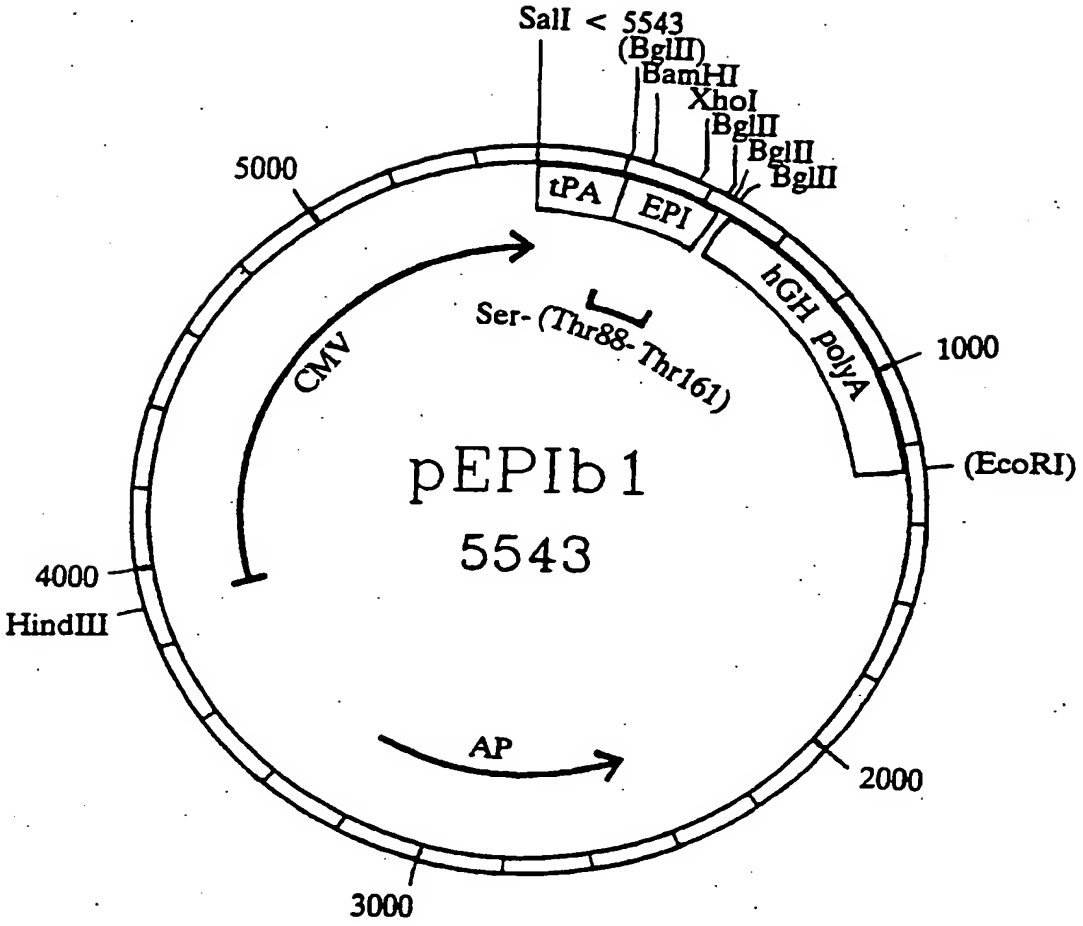
SalI < 5543
(BglII)
BamHI
XhoI
BglII
BglII
BglII
5000
tPA
EPI
hGH polyA
1000
(EcoRI)
CMV
Ser-(Thr88-Thr161)
pEPIb1
5543
4000
HindIII
AP
2000
3000

Fig. 5

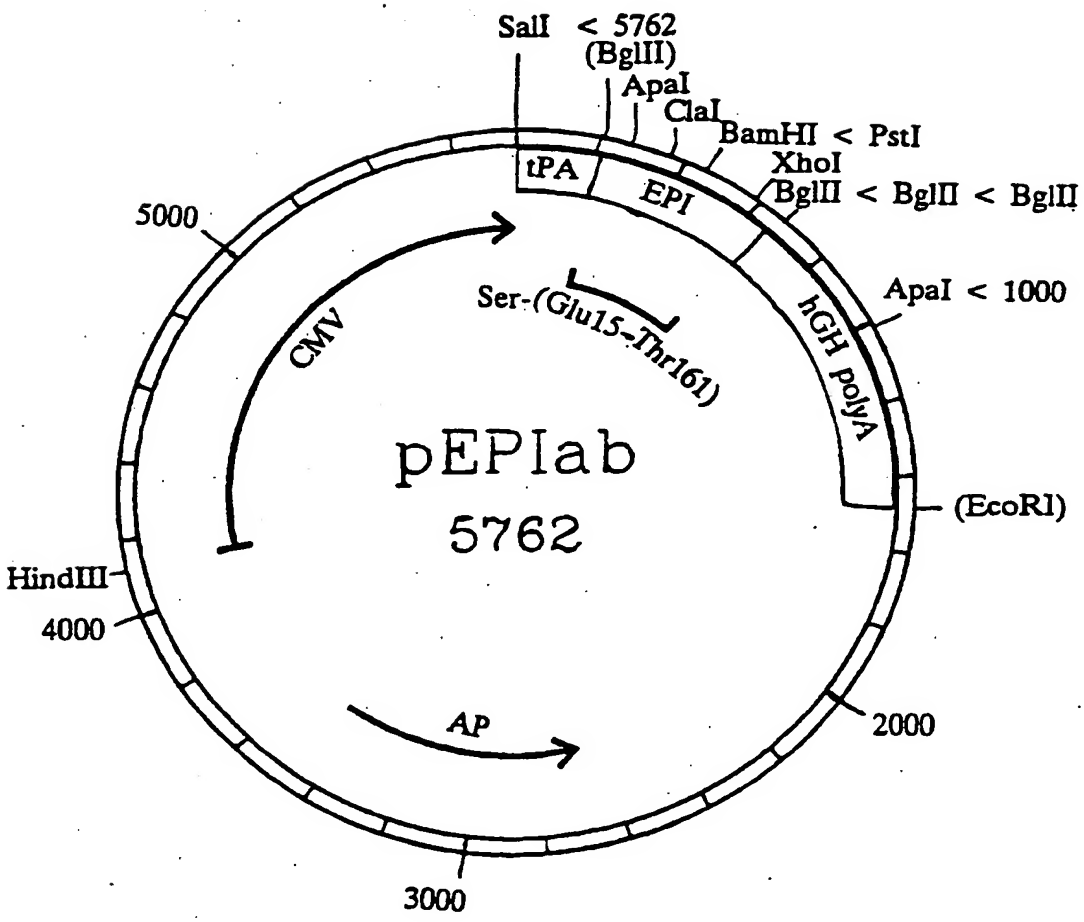
SalI < 5762
(BglII)
ApaI
ClaI
BamHI < PstI
XhoI
BglII < BglII < BglII
tPA
EPI
5000
Ser-(Glu15-Thr161)
CMV
hGH polyA
ApaI < 1000
pEPIab
5762
(EcoRI)
HindIII
4000
AP
2000
3000

Fig. 6

```
GTCGACAGAG CTGAGATCCT ACAGGAGTCC AGGGCTGGAG AGAAAACCTC        50

TGCGAGGAAA GGGAAGGAGC AAGCCGTGAA TTTAAGGGAC GCTGTGAAGC       100

AATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG      143
     Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu
      1               5                  10

CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT      185
Leu Cys Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His
    15                  20                  25

                                        PstI
GCC CGA TTC AGA AGA GGA GCC AGA TCA ACA CTG CAG CAA GAA      227
Ala Arg Phe Arg Arg Gly Ala Arg Ser Thr Leu Gln Gln Glu
        30                  35                  40

                                    BamHI
AAG CCA GAT TTC TGC TTT TTG GAA GAG GAT CCT GGA ATA TGT      269
Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys
            45                  50                  55

CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT CAG ACA AAA      311
Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys
                60                  65

CAG TGT GAA AGG TTC AAG TAT GGT GGA TGC CTG GGC AAT ATG      353
Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met
70                  75                  80

                    XhoI
AAC AAT TTT GAG ACA CTC GAG GAA TGC AAG AAC ATT TGT GAA      395
Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu
    85                  90                  95

                                        KnpI
GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT GGT ACC TGA      437
Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly Thr End
        100                 105                 110

BglII           BglII               BglII
AGATCTGAAT TCTGAAGATC TAGGCCTATG AAGATCT                     474
```

Fig. 7

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```
                SalI
GTCGACAGAG CTGAGATCCT ACAGGAGTCC AGGGCTGGAG AGAAAACCTC                    50

TGCGAGGAAA GGGAAGGAGC AAGCCGTGAA TTTAAGGGAC GCTGTGAAGC                   100

AATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG                  143
     Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu
      1               5                  10

CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT                   185
Leu Cys Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His
    15                  20                  25

                                    SacI
GCC CGA TTC AGA AGA GGA GCC AGA TCA GAG CTC CCA CCA CTG                   227
Ala Arg Phe Arg Arg Gly Ala Arg Ser Glu Leu Pro Pro Leu
        30                  35                  40

                                                    ApaI
AAA CTT ATG CAT TCA TTT TGT GCA TTC AAG GCG GAT GAT GGG                   269
Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly
            45                  50                  55

CCC TGT AAA GCA ATC ATG AAA AGA TTT TTC TTC AAT ATT TTC                   311
Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe
                60                  65

ACT CGA CAG TGC GAA GAA TTT ATA TAT GGG GGA TGT GAA GGA                   353
Thr Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly
70                  75                  80

          ClaI
AAT CAG AAT CGA TTT GAA AGT CTG GAA GAG TGC AAA AAA ATG                   395
Asn Gln Asn Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met
    85                  90                  95

                                                    PstI
TGT ACA AGA GAT AAT GCA AAC AGG ATT ATA AAG ACA ACA CTG                   437
Cys Thr Arg Asp Asn Ala Asn Arg Ile Ile Lys Thr Thr Leu
        100                 105                 110

                                                BamHI
CAG CAA GAA AAG CCA GAT TTC TGC TTT TTG GAA GAG GAT CCT                   479
Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro
            115                 120                 125

GGA ATA TGT CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT                   521
Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn
                130                 135
```

Fig. 8

```
CAG ACA AAA CAG TGT GAA AGG TTC AAG TAT GGT GGA TGC CTG          563
Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu
140                 145                 150

                                    XhoI
GGC AAT ATG AAC AAT TTT GAG ACA CTC GAG GAA TGC AAG AAC          605
Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn
    155                 160                 165

ATT TGT GAA GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT          647
Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr
        170                 175                 180

KpnI          BglII         BglII           BglII
GGT ACC TGA AGATCTGAAT TCTGAAGATC TAGGCCTATG AAGATCT             693
Gly Thr End
```

Fig. 8 cont.

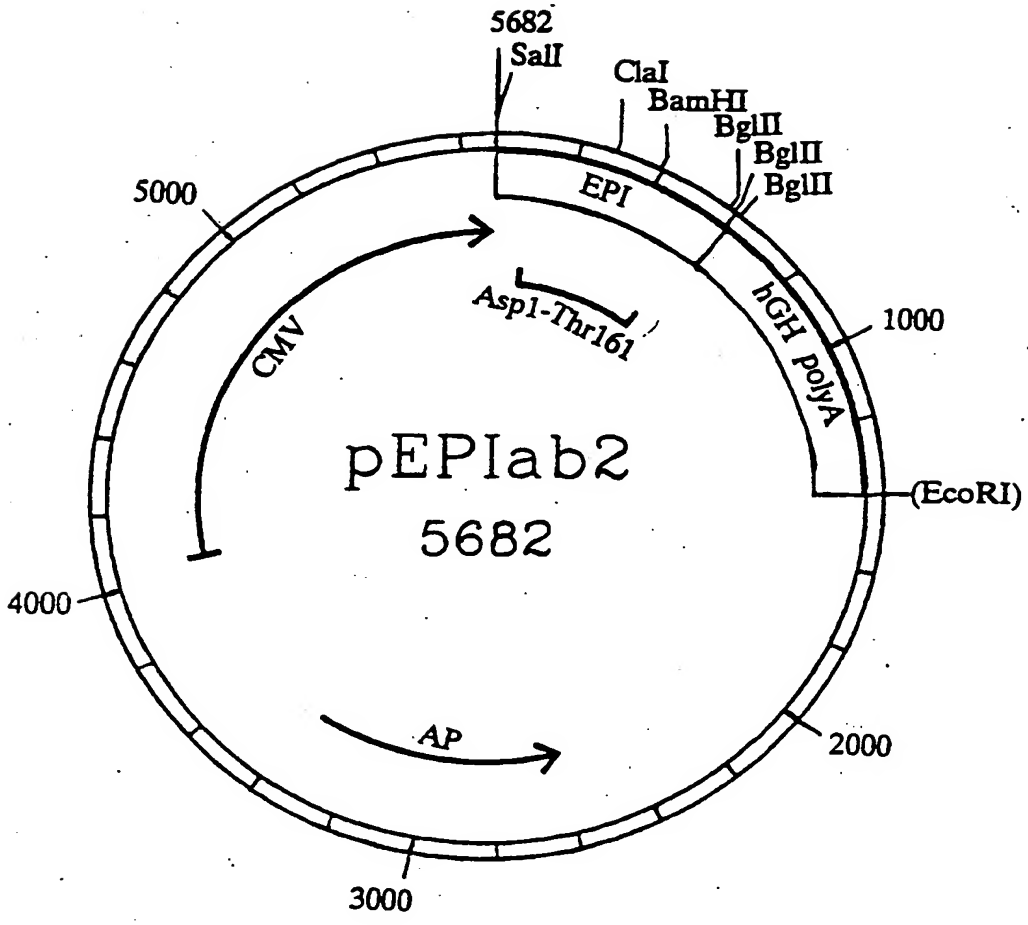

Fig. 9

REPLACEMENT SHEET

```
SalI
GTCGACC ATG ATT TAC ACA ATG AAG AAA GTA CAT GCA CTT TGG          43
        Met Ile Tyr Thr Met Lys Lys Val His Ala Leu Trp
         1               5                  10

GCT AGC GTA TGC CTG CTG CTT AAT CTT GCC CCT GCC CCT CTT          85
Ala Ser Val Cys Leu Leu Leu Asn Leu Ala Pro Ala Pro Leu
        15                  20                  25

AAT CGT GAT TCT GAG GAA GAT GAA GAA CAC ACA ATT ATC ACA         127
Asn Ala Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr
            30                  35                  40

GAT ACG GAG CTC CCA CCA CTG AAA CTT ATG CAT TCA TTT TGT         169
Asp Thr Glu Leu Pro Pro Leu Lys Leu Met His Ser Phe Cys
                45                  50

GCA TTC AAG GCG GAT GAT GGG CCC TGT AAA GCA ATC ATG AAA         211
Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala Ile Met Lys
55                  60                  65

AGA TTT TTC TTC AAT ATT TTC ACT CGA CAG TGC GAA GAA TTT         253
Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe
    70                  75                  80

                                        ClaI
ATA TAT GGG GGA TGT GAA GGA AAT CAG AAT CGA TTT GAA AGT         295
Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn Arg Phe Glu Ser
        85                  90                  95

CTG GAA GAG TGC AAA AAA ATG TGT ACA AGA GAT AAT GCA AAC         337
Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn Ala Asn
                100                 105

AGG ATT ATA AAG ACA ACA CTG CAG CAA GAA AAG CCA GAT TTC         379
Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe
110                 115                 120

                BamHI
TGC TTT TTG GAA GAG GAT CCT GGA ATA TGT CGA GGT TAT ATT         421
Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile
    125                 130                 135

ACC AGG TAT TTT TAT AAC AAT CAG ACA AAA CAG TGT GAA AGG         463
Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
        140                 145                 150

TTC AAG TAT GGT GGA TGC CTG GGC AAT ATG AAC AAT TTT GAG         505
Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu
            155                 160                 165
```

Fig. 10

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```
AGA CTC GAG GAA TGC AAG AAC ATT TGT GAA GAT GGT CCG AAT          547
Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly Pro Asn
                170                     175

                                                BglII
GGT TTC CAG GTG GAT AAT TAT GGT ACC TGA AGATCTGAAT               587
Gly Phe Gln Val Asp Asn Tyr Gly Thr End
180                 185

     BglII              BglII
TCTGAAGATC TAGGCCTATG AAGATCT                                    614
```

Fig. 10 cont.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00212

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 13/00, C 12 N 15/15, A 61 K 37/64

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N; A 61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Nature, Vol. 338, 1989 J. Girard et al.: "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor", see especially p. 518 col. 2 lines 11-12, p. 520 col 1 lines 1-8	1-3,14-16
Y	--	17
Y	EP, A2, 0318451 (MONSANTO COMPANY) 31 May 1989, see the whole document	17
	--	
A	EP, A2, 0300988 (WASHINGTON UNIVERSITY) 25 January 1989, see the whole document	1-18
	--	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th November 1990	1990 -11- 19
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Thrombosis research, Vol. 48, 1987 B.J. Warn-Cramer et al.: "Partial Purification and characterization of extrinsic ", see page 11 - page 22	1-18
A	Proc. Natl. Acad. Sci., Vol. 84, 1987 George J. Broze, Jr. et al.: "Isolation of the tissue factor inhibitor produced by HepG2 hepatoma cells ", see page 1886 - page 1890	1-18

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers....1....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claim states that the EPI analogue has a low heparin binding capacity, without describing anything about the structure of the analogue and hence, the claim is considered not to be clear and concise, c.f. PCT article 6.

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 90/00212

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 90-09-27
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0318451	89-05-31	AU-D-	1928788	89-05-25
		JP-A-	1165383	89-06-29
EP-A2- 0300988	89-01-25	AU-D-	1928688	89-02-02
		JP-A-	1047799	89-02-22